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Virology 334 (2005) 294–298

VIROLOGY

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HPV16, HPV18, and HIV infection may influence cervical cytokine intralesional levels

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Received 26 July 2004; returned to author for revision 19 November 2004; accepted 26 January 2005

Abstract

Infection with oncogenic human papillomavirus (HPV) is considered to be the major risk to cervical cancer. This study analyzed the influence of HPV infection on cytokine intralesional levels in cervical lesion in the presence or not of HIV infection. Cervical biopsies from 42 women were studied. HPV detection and typing were performed using amplified DNA hybridized with sequence-specific primers, and cytokine intralesional levels were detected using ELISA. HPV16+ biopsies exhibited increased IFN-γ and IL-10 when compared to HPV16− ($P = 0.03$ and 0.04, respectively). HPV18+ biopsies exhibited decreased TNF-α ($P = 0.009$) and IFN- γ ($P = 0.01$) when compared to HPV18−. In accordance to HIV status, HIV−/HPV16+ patients exhibited increased IFN-γ when compared to those presenting HIV−/HPV16− ($P = 0.007$). HIV−/HPV18+ patients presented decreased IFN-γ when compared to HIV−/HPV18− ($P = 0.02$). These results suggest that the presence of HPV16 infection may influence cervical lesion installation, and irrespective of HIV status, HPV18 infection may be more aggressive than HPV-16.

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Keywords: HPV; HIV; Cytokine; Cervical lesion

Introduction

Cancer of the cervix accounts for almost 12% of all cancers in women and represents the second most frequent gynecological malignancy in the world (Pisani et al., 2002). Infection with high-risk human papillomavirus (HPV) types, particularly HPV 16 and 18, is considered to be the major risk factor for cervical cancer development (Schiffman et al., 2000; Zur Hausen, 1999). Although in 70–90% of HPV-infected individuals the virus is usually cleared (Ho et al., 1998), in a small percentage of patients the virus persists and may lead to the development of high-grade squamous intraepithelial lesions (HSILs) (Zur Hausen, 2002).

Cytokines play an important role in the defense against HPV infection, modulating viral replication and polarizing the immune response to the Th1 or Th2 pattern (Zur Hausen, 2002). A predominance of Th1 polarization, with production of IFN-γ, has been associated with the clearance of HPV infection and regression of SIL (Stellato et al., 1997). In addition, in the presence of TNF-α, an antiviral effect with growth-inhibitory and virus-suppressive properties is also observed (Bachmann et al., 2002). In contrast, immunosuppressive cytokines (IL-10, TGF-β) are associated with the persistence of HPV infection and progression to SIL by suppressing cell-mediated immunity

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0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.01.029

(Clerici et al., 1997; Gravitt et al., 2003; Stanczuk et al., 2002).

Recent studies have reported an increased prevalence and persistence of HPV 16 and 18 in the genital tract of HIV-positive women. The predominance of Th2 cytokine polarization in HIV-positive women may explain, in part, the susceptibility to recurrent genital tract infections and SIL occurrence, permitting the development of neoplastic atypias (Ahmed et al., 2002; Gonçalves et al., 2003; Olaitan et al., 1998).

In the present study, we evaluated cytokine intralesional levels (TNF-α, IL-10, and IFN-γ) in women presenting with low-grade (LG) or high-grade (HG) SIL, infected with HPV, and presenting or not HIV infection as co-morbidity.

Results

Frequency of HPV and HIV infection

All patients analyzed were infected with HPV and were stratified in 8 subgroups, according to the HPV type and HIV infection:

a. HIV negative, HPV16+ (n = 11);
b. HIV negative, HPV18+ (n = 6);
c. HIV negative, HPV16+, HPV18+ (n = 3);
d. HIV negative, other HPV positive (n = 2);
e. HIV positive, HPV16+ (n = 10);
f. HIV positive, HPV18+ (n = 1);
g. HIV positive, HPV16+, HPV18+ (n = 2);
h. HIV positive, other HPV positive (n = 7).

HPV infection and intralesional cytokine levels

When patients were stratified according to HIV infection or SIL severity, no significant differences were found. Similarly, when HIV-positive patients were stratified according to the severity of the lesion and according to CD4 T-cell count, no significant differences were observed.

When patients were stratified according to HPV type, those presenting with HPV16 infection exhibited increased intralesional levels of IFN-γ and IL-10, compared to those without HPV16 infection (P = 0.03 and 0.04, respectively). On the other hand, patients presenting with HPV18 infection exhibited decreased TNF-α and IFN-γ intralesional levels when compared to those without HPV18 infection (P = 0.009 and P = 0.01, respectively).

To avoid the confounding influence of HPV 16 on HPV 18 and vice versa on the comparisons with patients who did not harbor these types, additional groups were assigned. Patients presenting exclusively with HPV 16 infection exhibited increased IL-10 levels when compared to those with other HPV types excluding HPV 18 (P = 0.008), whereas patients presenting exclusively with HPV18 infection exhibited IL-10 levels closely similar to those observed for other HPV types excluding HPV16 (P = 0.32). On the other hand, patients presenting exclusively with HPV18 infection exhibited decreased levels of TNF-α (P = 0.005) and of IFN-γ (P = 0.04) when compared with those presenting exclusively with HPV16 infection. Finally, patients presenting both HPV16 and 18 exhibited closely similar levels of TNF-α, IFN-γ, and IL-10 when compared to patients harboring other HPV types. The results that revealed significant associations regarding intralesional cytokine levels are shown in Table 1.

HPV and HIV infection associated with intralesional cytokine levels

When patients were stratified according to HIV status and HPV type, specimens collected from HIV+/HPV16+ patients exhibited increased IL-10 levels when compared to HIV+/HPV16− patients (P = 0.04), as illustrated in Table 2. Regarding HIV-negative patients, those presenting HPV16 infection exhibited increased intralesional IFN-γ levels compared to patients without HPV16 infection (P = 0.007). In addition, HIV−/HPV18+ patients presented decreased intralesional IFN-γ levels compared to HIV−/HPV18− patients (P = 0.02).

With regard to the severity of SIL and HPV type, patients presenting with HSIL/HPV16− showed a trend increase of

Table 1
Intralesional cytokine levels (pg/50 μL) stratified according to the type of HPV infection (16 or 18) and HIV status

Cytokine	HPV16		HPV18		HIV negative		HIV negative	
	Negative (n = 16)	Positive (n = 26)	Negative (n = 30)	Positive (n = 12)	HPV16− (n = 08)	HPV16+ (n = 14)	HPV18− (n = 13)	HPV18+ (n = 09)
IFN-γ	83	112	113	87	58	112	116	77
	(8–116)	(8–146) **0.03***	(8–146)	(8–132) **0.01***	(8–102)	(8–146) **0.007***	(8–146)	(8–102) **0.02***
TNF-α	4	8	14	0	4	0	8	0
	(0–59)	(0–85) 0.49*	(0–85)	(0–45) **0.009***	(0–19)	(0–76) 0.91*	(0–76)	(0–18) 0.12*
IL-10	30	227	125	51	41	142	139	51
	(0–415)	(0–390) **0.04***	(0–391)	(0–415) 0.97*	(0–415)	(0–391) 0.46*	(0–391)	(0–415) 1.00*

Median (range) values are also shown.
* P value.

Table 2
Intralesional cytokine levels (pg/50 μL) of TNF-α and IL-10 stratified according to the presence of HIV infection and to the absence of HPV16 infection

Cytokine	HIV positive		HPV16 negative	
	HPV16− (n = 08)	HPV16+ (n = 12)	LG-SIL (n = 09)	HG-SIL (n = 07)
TNF-α	5 (0–59)	4 (0–85) 0.41*	0 (0–12)	17 (0–59) **0.05***
IL-10	8 (0–337)	278 (0–338) **0.04***	15 (0–337)	51 (0–415) **0.60***

Median (range) values are also shown.
* P value.

TNF-α levels compared with those presenting LSIL/HPV16− (P = 0.05), as shown in Table 2.

Discussion

Several authors have reported that the immune response against HPV and HIV infection shows polarization to the Th2 cytokine profile, either locally or at the systemic level, facilitating neoplastic transformation and predisposing women to SIL and cervical cancer (Ahmed et al., 2002; Olaitan et al., 1998). To our knowledge, this is the first study that analyzes the intralesional cytokine levels in women presenting with HPV-infection, stratified according to HIV status and SIL severity.

IFN-γ mediates cellular immunity and is effective in host defense against viral infections and tumors, whereas IL-10 mediates humoral immunity against extracellular antigens and is immunoinhibitory, stimulating tumor growth (Clerici and Shearer, 1994; El-Sherif et al., 2001; Olaitan et al., 1998; Rengarajan et al., 2000). In this series, we observed that the presence of HPV16 was associated with increased IL-10 and IFN-γ intralesional levels when compared to patients who harbored other HPV types. Furthermore, the presence of an additional risk factor, i.e., the superimposed HIV infection (HIV+/HPV16+), was associated with increased intralesional levels of IL-10, whereas HIV−/HPV16+ patients presented increased IFN-γ levels. These findings support the idea that increased IL-10 in cervical lesions of HIV-positive women may facilitate neoplastic transformation in the presence of HPV16, which has long been considered an important agent endowed with a high oncogenic potential. These findings are in accordance with those reported by Crowley-Nowick et al. (2000) in cervico-vaginal secretions; however, the present study is the first to describe intralesional cytokine measurements in SIL fragments infected with HPV16 and HPV18. In addition, the findings of the present study regarding the increased IL-10 levels in biopsies from HIV-positive patients and increased IFN-γ levels in biopsies from HIV-negative patients corroborate the ample evidence in the literature that HIV-infected patients are at risk for HPV-16-associated dysplasia and malignancy based on prospective studies (Minkoff et al., 1998). Finally, the present results suggest that the intralesional cytokine profile may modulate anti-tumor activity, a finding that may have practical implications in the development of immunotherapy for cervical cancer, particularly for HIV-infected women. HPV18-infected women, irrespective of HIV status, showed reduced intralesional levels of TNF-α and INF-γ in relation to those who did not harbor HPV18, even after excluding patients presenting HPV 16. These findings differ from those observed for HPV16 patients of this series, suggesting that the HPV type may induce distinct immune inhibitory response, sometimes increasing IL-10 and sometimes decreasing TNF-α and IFN-γ. Corroborating this idea, when both HPV 16 and 18 were found together, no particular difference in terms of cytokine intralesional levels was observed when compared to other HPV types.

In contrast to HPV16, HPV18 has been reported to be integrated into the cell genome even in pre-neoplastic lesions, becoming more invasive, and possibly more aggressive (Badaracco et al., 2002). TNF-α represents a key regulatory cytokine involved in the regression of benign tumors and playing a pivotal role in the control of dysplastic cervical lesions infected with high-risk HPV types (Bachmann et al., 2002). In addition, TNF-α possesses growth-inhibitory functions in non-malignant HPV-infected keratinocytes, suppressing E6 and E7 viral oncogene transcription and inducing the expression of monocyte chemoattractant protein-1 (MCP-1) (Rösl et al., 1994). Cervical carcinoma cells are completely refractory to triggering MCP-1 gene expression, despite functional TNF-α signaling (Finzer et al., 2000). Furthermore, conversion to carcinogenicity is accompanied by resistance against TNF-α, a cytokine capable of selectively suppressing HPV18 transcription in formerly non-malignant cells (Soto et al., 1999). These literature findings together with the results of the present study suggest that HPV 18 may be more difficult to be cleared in relation to HPV-16.

In conclusion, the concomitance of HPV16 and HIV infection was associated with increased intralesional IL-10 levels. Similarly, the presence of HPV18, irrespective of HIV status, was associated with decreased TNF-α and IFN-γ intralesional levels. Taken together, these findings may contribute to SIL installation. Considering that this study was originally designed as a transversal study, further studies including larger number of patients should be performed to address the specific issue of how much the specific HPV types might influence the cytokine intralesional levels.

Materials and methods

Study population

Patients

Forty-two women aged 16–46 years (median = 27) presenting with distinct SIL grades were selected from

the Outpatient Gynecology Clinic of the University Hospital of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil, from October 2001 to November 2002. Twenty-two patients exhibited LSIL (10 HIV positive and 12 HIV negative) and 20 HSIL (10 HIV positive and 10 HIV negative). Of the LSIL group, 12/20 (60%) of patients reported to be current smokers and 2/20 (10%) were using oral contraceptive. Of the HSIL group, 10/13 (77%) patients reported to be current smokers and 1/13 (8%) was using oral contraceptives.

Twenty patients aged 16–40 years (median = 28) were infected with HIV and 22 aged 16–40 (median = 26.5) were not. According to the Centers for Disease Control and Prevention (CDC) classification (Centers for Diseases Control, 1992), 8 of the 20 HIV-positive patients (40%) were asymptomatic and 12/20 (60%) presented AIDS and were receiving highly active antiretroviral therapy (HAART) at the time of the study. Regarding the CD4 T-cell count, 5/20 (25%) exhibited counts higher than 500/μL, 13/20 (65%) between 500 and 200/μL, and 2/20 (10%) lower than 200/μL.

Ethical aspects

The Medical Ethics Committee of the University Hospital of Faculty of Medicine of Ribeirão Preto, Brazil, approved the study protocol (process number 6597/2001), and informed consent was obtained from all individuals.

Sample collection

Colposcopically directed cervical biopsies were collected and cervical specimens were cut in two sections, one fixed with formalin and stained with hematoxylin-eosin (Sigma, St. Louis, MO, USA) to analyze tissue morphology and integrity. Two pathologists blinded to one another reviewed all the histopathological diagnoses. Cervical biopsies from HIV-negative or HIV-positive women were stratified into 2 groups: LSIL (cervical intraepithelial neoplasia I and HPV DNA positive) and HSIL (cervical intraepithelial neoplasia II–III and in situ carcinoma). The other cervical fragment was immediately immersed in liquid nitrogen and maintained at −70 °C until use. Cervical DNA was used for HPV detection and typing, and cervical protein was used for the quantification of intralesional cytokine levels.

DNA and protein extraction

Intralesional protein and genomic DNA from cervical biopsies were extracted simultaneously using Trizol (GIBCO, RD, USA) according to manufacturer instructions and maintained at −70 °C until quantification.

Intralesional cytokine levels

A double-sandwich ELISA was performed to measure the concentration of TNF-α, INF-γ, and IL-10 in protein obtained from cervical biopsies, as previously described (Cunha et al., 2003), with minor modifications. Briefly, microtiter plates (Nunc-Maxisorb) were coated overnight at 4 °C with 50 μL (2 μg/mL) of antihuman antibody specific for these cytokines and washed three times with PBS containing 0.1% Tween 20 (Sigma). Wells containing blocking buffer (PBS plus 0.1% Tween 20 and 1.0% filtered BSA) were incubated for 2 h at 4 °C and then washed three times with PBS plus 0.1% Tween 20. Samples in a volume of 50 μL were added to each well. The plates were incubated overnight at 4 °C and then washed three times with PBS plus 0.1% Tween 20. Then, 50 μL (1 μg/mL) of a polyclonal anti-cytokine antibody conjugated with biotin diluted in blocking buffer was added to each well. The plates were then incubated for 1 h at 37 °C. After three washes with PBS and 0.1% Tween 20, the reaction products were detected with an avidin-biotin-peroxidase complex (vector). The color of the reaction was developed using *o*-phenylenediamine dihydrochloride (Sigma) and read at 490 nm. The concentrations of cytokines in the samples were calculated from a standard curve of human recombinant cytokine. Although fragment size was closely similar, we adjusted the cytokine concentration according to the DNA concentration obtained from the same fragment and the results were expressed as pg/50 μL.

HPV DNA detection and typing

DNA was amplified using 12.5 pM of each dNTP, 25 pM of each primer, 1.5 U Taq DNA polymerase (BioTools, Madrid, Spain), 5 μL of 10× enzyme buffer, 20 μg of genomic DNA, and distilled deionized water to complete a total volume of 25 μL. The mixture was processed in a thermocycler apparatus (MJ Research, MA, USA) under the following cycling conditions: 1 cycle at 95 °C for 5 min, then 30 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and finally 1 cycle at 72 °C for 10 min, and then, at 4 °C indefinitely.

Several pairs of primers were used. The primers GP5 and GP6 (Nijders et al., 1990), which amplify small DNA fragments, and the primers MY09 and MY11 (Manos, 1989), which amplify longer DNA fragments, were used for generic HPV amplification.

Since HPV 16 and HPV 18 are the types most frequently associated with SIL, attention was focused on them. The primers HPV16E7.667/HPV16E7.774 specific for HPV16 and HPV18E7.696/HPV18E7.799 (Walboomers et al., 1999) specific for HPV18 were also used, considered as positive only unambiguous amplifications. Lack of amplification with these primers or ambiguous amplifications after several repetitions was assigned as other HPV types.

Amplified DNA was applied to 10% polyacrylamide gel, electrophoresed at 200 mA for 2 h, and stained with $AgNO_3$ by the method of Sanguinetti et al. (1994).

Statistical analysis

According to the distribution of the data concerning the intralesional cytokine levels, the unpaired *t* test or the Mann–Whitney test (MW) was used. The Instat program (GraphPad software, CA, USA) was used for all these analyses. In a 95% confidence interval, *P* values less than or equal to 0.05 were considered to be significant.

Acknowledgments

This research was supported by FAPESP (Gonçalves, MAG: 97/04490-8 and 01/02908-2).

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